Improvement of an expression vector for production of recombinant proteins

Insal al 7

Field of the invention.

The present invention relates to DNA plasmids to be used for the production of recombinant proteins. More specifically, the present invention concerns the addition of specific DNA elements to expression plasmids that serve a function as enhancing elements. The outcome is to improve the yields of recombinant protein production.

Background of the invention.

There are a number of different strategies for the large-scale production of recombinant proteins to be used in, for example, the pharmaceutical industry. In certain cases it is desirable that the recombinant protein is made in eucaryotic hosts. These hosts may be cultivated cells or animals made transgenic with respect to the gene of interest. In the latter situation, transgenic expression in milk is a valuable technique since transgenes, active in the mammary gland, have been described and milk is a readily available body fluid.

The present invention relates to, in an unrestricted way, an improvement in expression vectors used to produce recombinant proteins in milk. These improved expression vectors will increase the yield of valuable recombinant proteins which will be of value for the facilitation of subsequent handling and purification steps.

Construction of a transgene requires certain basic ingredients, one being the structural gene containing the coding information for the protein of interest. A basal eucaryotic gene expression promoter is also required. In addition, other sequences can be used that confer tissue specificity or enhance expression in response to stimulus. The present invention relates to a specific type of enhancers, namely enhancers responding to hormonal stimuli. The particular enhancer in question is a sequence of DNA that confers a response to signals

evoked by pituitary hormones belonging to the group of lactogenic hormones such as prolactin (Prl) and placenta lactogen (PL) and somatogenic hormones such as growth hormone (GH). Both of these groups of hormones occupy central roles in the stimulation of mammary gland development and function. The present invention concerns the definition of enhancers responding to both lactogenic and somatogenic hormones and the construction of expression vectors, that, in their ability to respond to both lactogenic and somatogenic hormones, will function in an improved manner as transgenes for production of recombinant proteins in milk.

Previous studies have defined a gene, the Serine Protease Inhibitor 2.1 (SPI) gene, that responds to GH. In the 5' flank of this gene a DNA element has been identified that enhances gene expression in a GH-dependent fashion. The sequence of this GH response element (SPI GH-RE) in question is: SEQ ID NO:1
GATCTACGCTTCTACTAATCCATGTTCTGAGAAATCATC
CAGTCTGCCCATG, (Yoon et al. J. Biol. Chem. 265; 19947 (1991))
Within this sequence we now disclose a shorter "SPI-GAS like element"; TTCTGAGAA, that constitutes the core GH regulated sequence. As exemplified below the SPI-GAS element is also functional when transferred to a reporter gene such as the Luciferase gene (Sliva D. et al J. Biol.. Chem. in press). In the following we also disclose that the GH-regulated sequences described above are also regulated by prolactin and that this can be used to design new expression vectors that improve exisitng vectors used to produce recombinant proteins in milk.

Examples

Example 1. Identification of a core GH regulated sequence.

The 50 bp SPI-GHRE: SEQ ID NO:1
(GATCTACGCTTCTACTAATCCATGTTCTGAGAAATCATC
CAGTCTGCCCATG) was used to identify a core GH regulated sequence using gel electrophoresis mobility shift assay (GEMSA). Nuclear extracts were prepared and incubated with a 32P labelled 50 bp SPI-GHRE. Subsequently the extracts were analysed on polyacrylamide gels. The results showed that nuclear proteins, dependent on GH, bound to this DNA sequence. By competition with shorter oligonucleotides derived from SPI-GHRE a core GH sequence was identified. Based on certain sequence homologies to interferon response elements we called this sequence SPI-GAS and also demonstrated that SPI-GAS functions as a GH regulated DNA

element when put into a reporter vector. The core SPI-GAS has the following sequence; TTCTGAGAA.

Example 2. Prolactin and growth hormone both activate SPI-TK-reporter gene.

An expression plasmid containing a recombinant hormone responsive reporter consisting of six repeats of a 50 bp growth hormone responsive element (GH-RE) from the serine protease inhibitor (SPI) 2.1 promoter fused to the thymidine kinase (TK) promoter was constructed. Corresponding constructs were made using the SPI-GAS element. Variants expressing either the bacterial protein chloramphenicol acetyl transferase (CAT) or firefly luciferase (SPI-CAT or SPI-Luc respectively) cDNAs were then constructed. Techniques to make these vectors are well known to experts in the field. The plasmid DNA constructions were transfected, together with plasmid expression vectors encoding either rat growth hormone receptors or mouse prolactin receptors, into Chinese hamster ovary (CHO) , COS, and Buffalo rat liver (BRL) cells, using DOTAP liposomes and according to the manufacturer instructions. Cells were incubated overnight with DNA and DOTAP in serum free media,left and then exposed to growth hormone or prolactin for 12 hours. Cell lysates were then prepared and CAT or luciferase enzyme activity measured. Both growth hormone and prolactin treatment lead to an approximately 5-fold stimulation reporter enzyme expression relative to transfected but non-hormone treated cells. These results show that both growth hormone and prolactin can regulate the reporter construct and that a requisite for this is the presence of SPI elements. The core element in the SPI-TK -reporter gene that confers GH regulation is likely to be; TTCTGAGAA, and similar results can be obtained with this element termed SPI-GLE as with the longer, 50 bp element named SPI-GHRE.

Example 3. Multimeric SPI elements in front of a TK promoter give a better response.

Reporters plasmids containing one to six copies of the 50bp SPI element fused to the TK promoter were constructed. The growth hormone responsiveness of these constructs was tested by transfection into a CHO cell line that stably expresses the rat growth hormone receptor DNA. Growth hormone stimulation of these cells showed that multimerization of SPI elements resulted in a larger growth hormone response.

Example 4. Expression of stable incorporated SPI -TK- Luciferase is growth hormone regulated.

To demonstrate that SPI elements retain growth hormone responsiveness function when genomically integrated CHO cells were transfected with the three following plasmids: SPI-LUC (described in example 1), an expression vector containing the CMV promoter and rat growth hormone receptor cDNA and a neomycin expression vector. Neomycin resistant clones were tested for growth hormone response by exposing cells to growth hormone for 12 h under serum free conditions and then measuring luciferase activity in cell lysates. The results indicated a three-fold growth hormone-regulated induction of expression of the stably integrated reporter gene.

Example 5. SPI elements in front of a strong promoter (SV40) results in a protein production that is further enhanced by GH.

Six copies of the SPI element were introduced upstream of a strong CMV promoter driving expression of the CAT cDNA in a plasmid construct. This construct was transfected into CHO-4 cells and GH regulation was tested as described above. It was found that GH stimulated the production of CAT.